Immunogenicity and Protective Efficiency in Mice of a Smallpox DNA Vaccine Candidate

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Abstract

The adverse reactions of the current live smallpox vaccine, and potential use of smallpox as a bioterrorism weapon, have highlighted the need to develop a new effective vaccine for this infectious disease. In the present study, a DNA vaccine vector was produced, which was optimized for expression of the vaccinia virus L1 antigen in a mouse model. Plasmid-encoded IgM-tL1R, which contains a truncated L1R gene fused to an IgM signal sequence, was constructed and expressed under the regulation of an SV40 enhancer. The expressed recombinant tL1 proteins were successfully secreted into the culture media. The DNA vaccine was administered to mice by electroporation, and animals were subsequently challenged with the lethal dose of vaccinia virus. We observed that immunization with IgM-tL1R induced robust neutralizing antibody responses and provided complete protection against a vaccinia virus infection. Isotyping studies revealed a lower IgG1/IgG2a ratio following vaccination with IgM-tL1R, suggesting the stimulation of Th1 immune responses. Our results propose that an optimized DNA vaccine, IgM-tL1R, can be effective in eliciting an anti-vaccinia virus immune response and provide protection against lethal orthopoxvirus challenge.

Introduction

Smallpox, a disease caused by variola virus, has been eradicated from the planet in 1980s following a global immunization program conducted by the World Health Organization. However, there are concerns that the smallpox virus could be used as a biological weapon. In addition, the monkeypox virus, which resembles the smallpox virus, is an emerging virus that caused an outbreak of human monkeypox in Africa, and more recently in the United States. Therefore, the need for protection against these poxviruses infections remains [1-3].

Variola belongs to the *Orthopoxvirus* genus, family *Poxviridae*, which also includes vaccinia, monkeypox, cowpox, and other viruses. Because the majority of orthopoxvirus DNA is highly conserved, considerable cross-protection can be conferred, through infection or immunization, within this family of viruses. For example, variola virus shares >90% similarity with the vaccinia genome, which enabled eradication of smallpox using the vaccinia virus. Orthopoxviruses contain a large linear double-stranded DNA genome encompassing, approximately 200 open reading frames, and replicate entirely in the cytoplasm [4,5]. Orthopoxviruses have two infectious form, the intracellular mature virion (IMV), and the extracellular enveloped virion (EEV). The IMV particles are assembled in a specific location in the cytoplasm and are trafficked by microtubules to sites of early endosomes or Golgi compartments, where they acquire an additional double-layer membrane, to from the EEV. Thus, the IMV and EEV are surrounded by a different number of lipid membranes. The IMV form has a role in infection transmission between hosts, whereas the EEV is necessary for long-range dissemination within the host [6].

The smallpox vaccines, Dryvax, and ACAM2000, are based on a live vaccinia virus. Dryvax was a first-generation smallpox vaccine, and one of the vaccines used during the worldwide eradication of smallpox. This vaccine was prepared by harvesting live vaccinia virus from lesions on the skin of infected cows. Although Dryvax was highly effective, it had adverse side effects including fever, headache, and myalgia. More serious side effects included generalized vaccinia, eczema, encephalitis, and even fatality [7,8]. Later, a second-generation vaccine, ACAM2000, was developed, focusing on cell culture methods for vaccine production. Regarding immunogenicity, this vaccine had comparable immunogenicity to that of the first-generation vaccine. However, ACAM2000 still had serious adverse events such as cardiac complications [9]. To overcome the side effects caused by the use of live-virus vaccines, protein- or DNA-based subunit vaccines are currently under investigation [10,11].

Subunit vaccines present only an antigen to the immune system, instead of the whole organism. Several vaccinia virus proteins were previously shown to be important antigens during orthopoxvirus infections. The viral protein components of IMV and EEV membranes are different and therefore present different target antigens. It is generally accepted that anti-IMV antibodies would act to block early entry of the virus and anti-EEV antibodies could prevent dissemination within the host [12].

The vaccinia virus L1 protein is encoded by the L1R gene and is highly conserved among orthopoxviruses [13]. L1 is a myristoylated 23-29 kDa transmembrane protein associated with IMV membrane, and is thought to have a role in IMV assembly or entry [14,15]. Previous studies showed that multicomponent vaccines that comprised antigens from the vaccinia virus membrane provided satisfactory protection against orthopoxvirus infections in experimental animals. One key component of these multicomponent vaccines is the IMV

membrane protein L1. For example, mice have been fully protected by a vaccine containing a combination of vaccinia L1, A33, and B5 proteins [16]. Similarly, Hooper et al. showed that gene gun delivery of DNA vaccines comprised of multiple plasmids encoding L1, A33, B5, and A27 protects mice against a lethal vaccinia virus challenge [17]. However, studies testing both protein- and DNA-based multicomponent vaccine immunizations indicated that vaccination with L1 alone provided only partial protection against disease [16,18].

In the present study, we modified the vaccinia virus L1R gene to gain more knowledge about immunity to smallpox. Here we report the results of our study using a DNA vaccine containing a modified L1R gene construct, delivered by intramuscular electroporation.

Materials and Methods

Viruses and cells

Vaccinia virus strain Western Reserve (WR) (ATCC VR-1354) was grown in HeLa cells and purified by sucrose density centrifugation. Viral concentrations were determined by plaque assays. For plaque reduction neutralization assays, 143 tk- cells were used. 293T cells were used for transient expression experiments. Both 293T and 143 tk- cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin) at 37°C in a humidified 5% CO_2 incubator.

Generation of a modified L1R gene

The L1R gene sequence of vaccinia virus (Genbank accession number NC_006998) was codon-optimized with commonly used codons in the human genome using the OptimunGeneTM by Genescript Corporation (Piscataway, USA). The IgM signal sequence was inserted at the N-terminus of the L1R gene, which was truncated after codon 185 (tL1R). The modified L1R gene was chemically synthesized (GeneScript).

Plasmid constructions

The DNA vaccine used in these studies, referred to as IgM-tL1R, is a plasmid expressing the C-terminal truncated L1 protein (tL1) of vaccinia virus. Modified L1R was cloned into the mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, USA), which utilizes the CMV promoter for expression. A codon-optimized truncated L1R gene was fused to the nucleotide sequence encoding the IgM signal sequence. pcDNA 3.1 was cleaved with *Eco*RI and *Apa*I, and the 618 bp truncated L1R gene containing the signal sequence was inserted into corresponding vector sites to obtain a modified L1 protein expression vector. The resulting vector also contained the enhancer from simian virus 40 (SV40). A single copy of a 72-bp element from the SV40 enhancer was inserted into a *Bgl*II/*Nru*I site located upstream of the coding region of the vector. This construct was referred to as IgM-tL1R. We also cloned the tL1 gene into the mammalian expression plasmid, pEGFP-C1 (Clontech, Mountain View, USA), which encodes an enhanced green fluorescent protein (EGFP). The codon-optimized tL1R gene was inserted into this vector using the restriction sites *Eco*RI and *Apa*I to create pEGFP/tL1R.

Analysis of tL1R expression

To confirm the expression of tL1R, 293T cells were cultivated in 6-well culture plates at a density of 1×10^6 cells per well in DMEM containing 10% FBS. Cells were transfected with one μg of plasmid DNA using Vivagen (Vivagen, Seoul, Korea) under conditions suggested by the manufacturer. After 48 h, cells and cell culture supernatants were collected. Samples were separated by 15% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked by incubation with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and probed for 1.5 h with primary mouse anti-tL1 polyclonal antibody (1:5000) followed by horseradish peroxidase-labeled anti-mouse antibodies (Santa Cruz Biotech, Santa Cruz, USA). Proteins were visualized using Western Lighting Plus-ECL (PerkinElmer, Waltham, USA). Expression of EGFP-tL1 fusion proteins was detected using a confocal laser-scanning microscope (LSM 5; Carl Zeiss, Oberkochen, Germany) or an immunoblot assay. Histidine-tagged recombinant tL1 protein produced in *Escherichia coli* and purified on nickel column was used to generate mouse anti-tL1 polyclonal antibody.

DNA vaccination

Six- to eight-week-old female BALB/c mice (Samtako, Osan, Korea) were anesthetized intraperitoneally with Zoletil at a dose of 2 mg/kg before DNA vaccine administration. The skin overlying the tibialis anterior muscle was shaved, and the mice were vaccinated three times at 2-week intervals, each with a dose of 50 μg of IgM-tL1R. Two-needle array electrodes with a 5 mm distance (BTX, Hollison, USA) were inserted into the muscle for electroporation immediately after DNA delivery. Three pulses of 90 V with 25 ms pulse length were delivered utilizing a BTX ECM-830 electroporation generator (Hollison, USA).

ELISA for measuring serum antibody

Mouse serum samples were collected every two weeks after each vaccination by retro-orbital bleeding and stored at -20°C until the assay. Briefly, microtiter plates were coated with *Escherichia coli*-expressed recombinant tL1 protein (1 μg/ml) in PBS overnight at 4°C and blocked with 2% skim milk buffer for 1 h at 37°C. The HRP-conjugated goat-anti mouse IgG was added to each well as a secondary antibody and incubated for 1 h at 37°C. The antibody titers were determined using an end-point dilution ELISA titration assay described before [19].

IgG isotype ELISA

Antibody isotypes were assessed using an Isotyping Kits for Immunoglobulins (eBioscience, San Diego, USA) according to the manufacturer's protocol. Plates were read by a Microplate Spectrophotometer (Molecular Devices, USA) at a wavelength of 450 nm.

Plaque reduction neutralization assay

Plaque reduction neutralization assays were described previously [16]. Neutralization assays were performed by pre-incubating 20 μl of vaccinia virus-infected cell lysates (200 PFU/ml) with mouse serum (1:10 dilution) at 37°C. Virus samples were then used to infect 143 tk-cell monolayers in 100 mm plates. After 2 h incubation, cells were washed with PBS and placed at 37°C with 5% CO_2. After 2 d, cell layers

stained with 1 ml of staining solution (0.1% crystal violet in 20% ethanol). After 30 min incubation crystal violet containing medium was removed from cells and the number of plaques could be counted. The neutralization is expressed as the percentage of reduction of plaque number versus the control, that the viral inoculum was incubated without serum.

Viral challenges

Two weeks after the final immunization, BALB/c mice (n=5/group) were intranasally challenged with 1×10^6 PFU of vaccinia virus strain WR. The control group received the injection with empty pcDNA 3.1. The mice were monitored for 12 d post-challenge to determine the status of vaccine protection. All animal experiments were performed in accordance with institutional guidelines of the University. Moribund mice (displaying a body weight loss of more than 30%) were euthanized.

Statistical analysis

Values for the mean and standard error of the mean were calculated for each data set. A Student's t test was used to determine statistical significance. P<0.05 was considered significant.

Results

Vaccine design

It has been demonstrated that recombinant truncated L1 protein was able to produce neutralizing antibodies against the vaccinia virus [20]. Truncated L1R encoded the first 185 amino acids, which corresponds to the cytoplasmic domain, and lacks the transmembrane domain. Therefore, we choose the truncated L1 protein (tL1) as a target antigen in the development of DNA vaccine. Previous our DNA vaccination studies indicate that efficiency of DNA vaccination can be improved by codon optimization and attachment of an IgM signal peptide sequence [19]. In addition to the use of modified antigen, the inclusion of transcriptional enhancers in the plasmid vector also has been shown to increase of DNA vaccine immunogenicity. Therefore, our final DNA vaccine construct included the 72-bp SV40 enhancer upstream of the codon-optimized tL1R coding region fused to an IgM signal peptide. This final DNA vaccine construct was referred to as IgM-tL1R.

In vitro expression of tL1 protein

The *in vitro* expression of tL1 protein was evaluated in 293T cells transiently transfected with the plasmid encoding tL1R. To confirm the transfection efficiency of the codon-optimized tL1R gene, we first constructed the eukaryotic expression vector, pEGFP/tL1R, encoding GFP-tagged tL1R. This vector was transfected into 293T cells. Expression of GFP gene was assessed by fluorescence microscopy 48 h after transfection. Fluorescence signals were visualized in cells transfected with codon-optimized tL1R but not in vector-only control cells (Figure 1A). In addition, immunoblot analysis confirmed a clear expression and detection of EGFP-tL1 fusion protein. The cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies directed against either GFP or tL1. As shown in Figure 1B, strong immunoblot signals were observed in EGFP or EGFP-tL1R transfected cells. As expected, the cells carrying pEGFP-tL1R expressed the fusion proteins of the predicted size which was 47 kDa, indicating that EGFP fusion protein can be efficiently expressed in our transfection system.

However, as shown in EGFP band in lane 3, proteolytic cleavage of the fusion proteins was also observed. Further cellular expression was certified by western blot analysis. IgM-tL1R was transfected into 293T cells and the expression of tL1, exhibiting the predicted molecular weight, was confirmed (Figure 1C). A protein of approximately 21 kDa, corresponding to the L1 cytoplasmic domain fused to a 20-amino-acid IgM signal peptide was detected in IgM-tL1R transfected cell lysates. In addition, we also observed additional non-specific bands due to the use of antiserum antibodies in western analysis. As expected, tL1 proteins were also detected in the supernatants of cells transfected with plasmid IgM-tL1R, which indicate that the signal peptide was indeed targeted tL1 for secretion. However, recombinant IgM-tL1 was secreted as lower molecular weight than cell-associated IgM-tL1, which seems to be that IgM signal peptide was cleaved during the secretion of mature protein into the medium. Previous study reported that the cleavage of IgM signal peptide is an important step for protein secretion in mammalian cells as well as in insect cell expression system [21]. No tL1-specific band was observed in extracts or supernatants of cells transfected with the control vector (pcDNA 3.1).
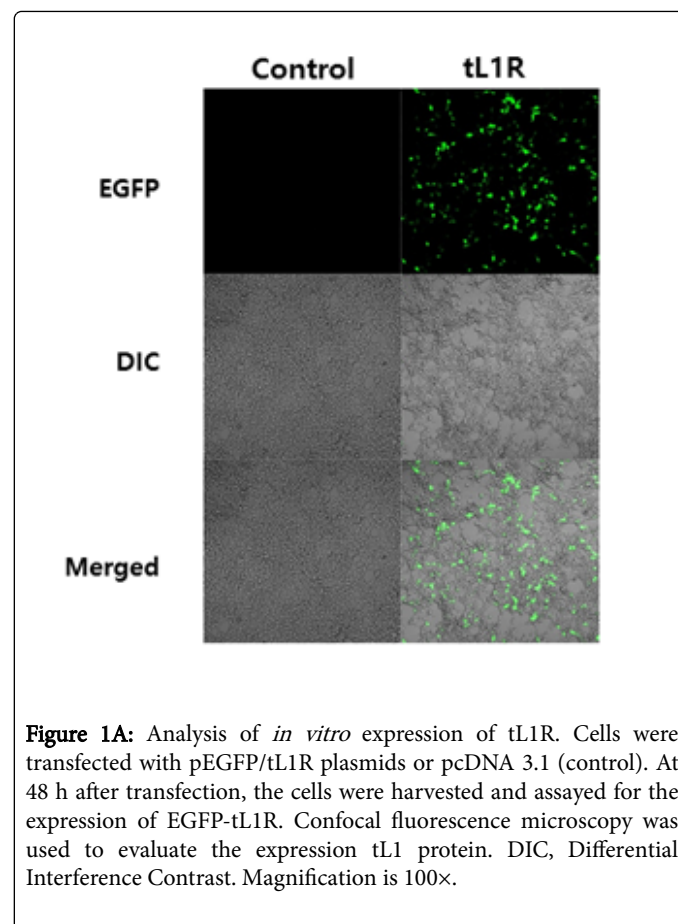

Figure 1A: Analysis of *in vitro* expression of tL1R. Cells were transfected with pEGFP/tL1R plasmids or pcDNA 3.1 (control). At 48 h after transfection, the cells were harvested and assayed for the expression of EGFP-tL1R. Confocal fluorescence microscopy was used to evaluate the expression tL1 protein. DIC, Differential Interference Contrast. Magnification is 100×.

Immunogenicity in mice

In vivo electroporation has been shown to enhance the transfer of DNA vaccines in various animal species [22]. Furthermore, recent clinical studies suggest that electroporation of a DNA vaccine is able to induce a robust immune response in patients [23]. Therefore, we used this method of delivering our IgM-tL1R plasmid to mice. To assess the immune responses induced by plasmid DNA immunization, female

BALB/c mice (n=5) were immunized by intramuscular injection with subsequent electroporation. Mice were vaccinated three times at 2-week intervals with the IgM-tL1R plasmid. A control group received the pcDNA 3.1 vector with no insert. ELISA was used to test for the presence of anti-tL1 IgG antibodies in serum samples obtained one week after each vaccination. As shown in Figure 2A, mice that were vaccinated with a 50 μg dose of IgM-tL1R had a dramatic increase in anti-tL1 IgG over the three weeks following the first injection. However, no significant anti-tL1R IgG levels were detected in the serum samples collected from control mice. As shown in Figure 2B, when 50 μg of DNA was delivered by electroporation, high antibody titers were observed compared to the control group ($p<0.001$). Taken together, these results indicate that electroporation-based vaccination with IgM-tL1R plasmid DNA rapidly induces a tL1-specific antibody response. In addition, DNA vaccination did not result any weight loss indicating that the mice tolerated the vaccination (Figure 2C). These results suggest that our DNA vaccine is safe and immunogenic in an animal model.
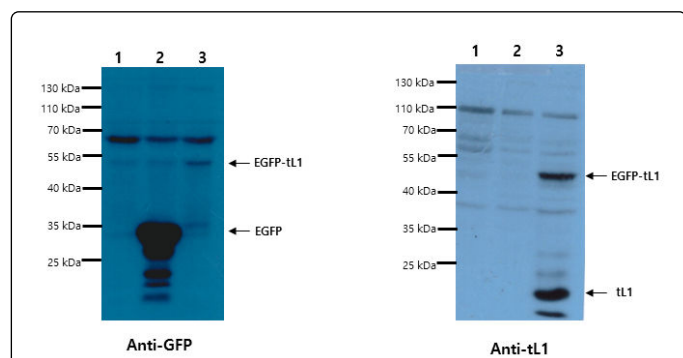

Figure 1B: Immunoblot analysis of EGFP-tL1 fusion proteins. Cells were transfected with plasmids expressing either EGFP-tL1 protein or EGFP. Immunoblots were analyzed with anti-GFP antibody (1:2000) or anti-tL1 antibody (1:5000). Lane 1, vector only control cell lysates; lane 2, EGFP-transfected cell lysates; lane 3, EGFP-tL1R transfected cell lysates.
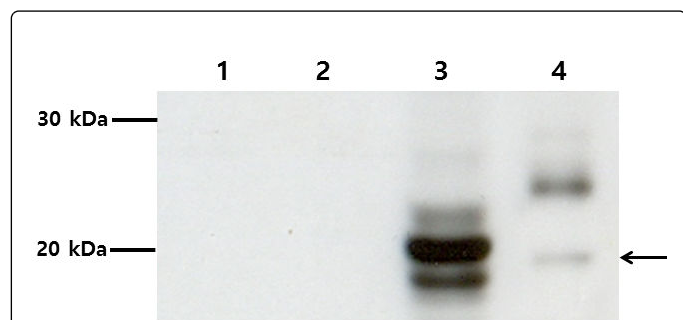

Figure 1C: Western blot analysis demonstrated the expression of IgM-tL1R. The proteins were detected by SDS-PAGE using a mouse polyclonal antibody against tL1. Lane 1, vector only control cell lysates; lane 2, vector only control cell supernatants; lane 3, IgM-tL1R transfected cell lysates; 4, IgM-tL1R transfected supernatants. Arrow indicates the position of the IgM-tL1R.
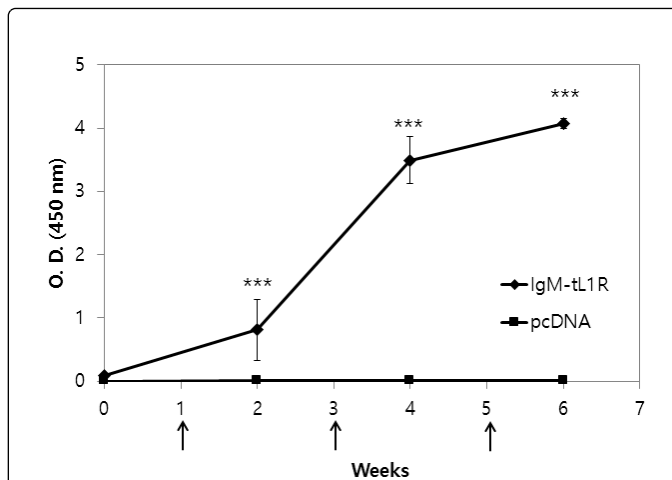

Figure 2A: tL1 specific antibody responses in mice immunized with IgM-tL1R. tL1 specific antibodies induced by DNA vaccine administration. Mice were immunized three times with IgM-tL1R DNA vaccine as indicated. Antibody levels are expressed as OD 450 values after the serum samples were diluted 100-fold. Values represent the mean ± standard deviation (asterisk denotes $p<0.001$ versus vector only control group).
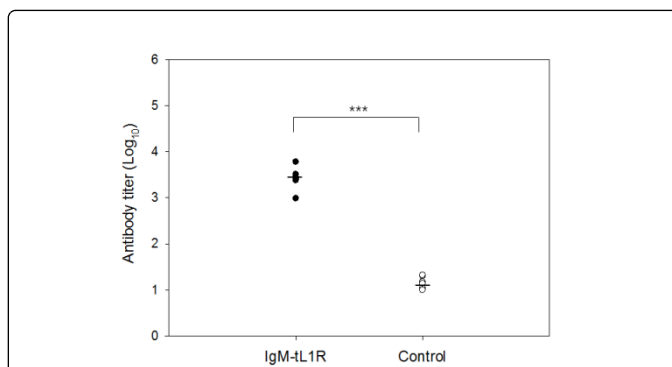

Figure 2B: Antibody levels are expressed as endpoint titers obtained using the ELISA assay. Mice were immunized three times at two-week intervals with the IgM-tL1R DNA vaccine, and serum samples were collected one week after the final immunization. Symbols show individual values; the bars represent mean titer for each group. Significant results are marked with asterisks: *** $p<0.001$.

Neutralization activities of antibodies

Since high levels of tL1-specific antibodies were detected in the serum samples of IgM-tL1R plasmid immunized mice, we next examined the neutralizing immune response using a plaque reduction assay. Neutralization assays were performed to evaluate if serum samples from immunized mice could prevent vaccinia virus infection in cultured cells. The virus neutralizing activity of serum samples (1:10 dilution) was measured before the challenge. Although some level of neutralizing activity was observed in the pre-immune group, a significant level of neutralizing activity (90% plaque reduction) was seen with serum samples from mice immunized with the IgM-tL1R plasmid (Figure 3). Pre-immunization serum samples were obtained

from mice prior to vaccination. Based on these results, it is clear that a neutralizing antibody response is elicited when mice are vaccinated with IgM-tL1R plasmid.
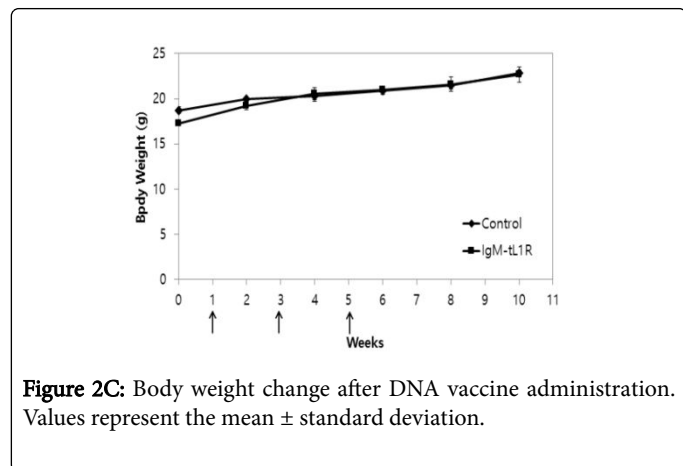

Figure 2C: Body weight change after DNA vaccine administration. Values represent the mean ± standard deviation.
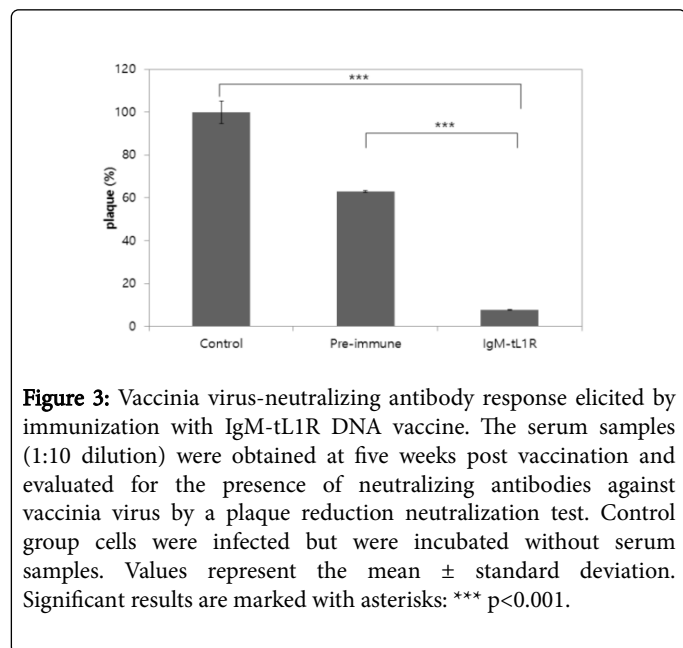

Figure 3: Vaccinia virus-neutralizing antibody response elicited by immunization with IgM-tL1R DNA vaccine. The serum samples (1:10 dilution) were obtained at five weeks post vaccination and evaluated for the presence of neutralizing antibodies against vaccinia virus by a plaque reduction neutralization test. Control group cells were infected but were incubated without serum samples. Values represent the mean ± standard deviation. Significant results are marked with asterisks: *** $p < 0.001$.

Antibody isotype profiles elicited by DNA vaccines

To screen the antigen-specific response provided by immunization with IgM-tL1R, both Th1 and Th2 type immune response were evaluated in immunized mice. The subclass distribution of serum IgG antibodies was analyzed over the course of immunization and was used as an indicator of Th1 or Th2 bias immune response induced (Figure 4A). It is known that IgG1 is associated with a Th2-like response, while a Th1 response is associated with the induction of other subclasses [24]. Thus, IgG1/IgG2a ratio was used as indicators of Th1 or Th2 polarized responses [25,26]. As shown in Figure 4B, IgG1/IgG2a ratios were relatively small in the immunized group (<1.0) compared to those of the pre-immunized group (>1.0). These results illustrate that vaccination with IgM-tL1R induces Th1-skewed immune with antibody.

Protection against lethal challenge with vaccinia virus

To test if vaccination with IgM-tL1R plasmid protects against viral infection, we performed *in vivo* experiments. BALB/c mice (n=5) were immunized intramuscularly with 50 μg of IgM-tL1R. The mice received two boosters with the same dose at 2-week intervals. Two weeks after the last immunization, we subjected mice to intranasal infection with 1×10^6 PFU of vaccinia virus-WR (a dose corresponding to 50 LD_{50}) [27]. Mice were then monitored for survival and body weight loss for 12 days following exposure. All mice that were immunized with the DNA vaccine survived lethal virus challenge (Figure 5A). As expected, all unvaccinated mice died within 6 to 7 d post-viral challenge. Although all immunized mice survived the virus challenge, they exhibited continuous body weight loss up to 6 d post-challenge (Figure 5B). However, they regained their starting weight between 7 and 12 d postinfection. Mice immunized with the DNA vaccine experienced weight loss that reached a maximum of 25% by day 6, whereas unimmunized mice lost as much as 32% of their starting weight by day 6, at which time all unimmunized mice died. Taken together, our results suggest that despite the transient weight loss, IgM-tL1R DNA vaccine immunization can protect mice against lethal vaccinia infections.
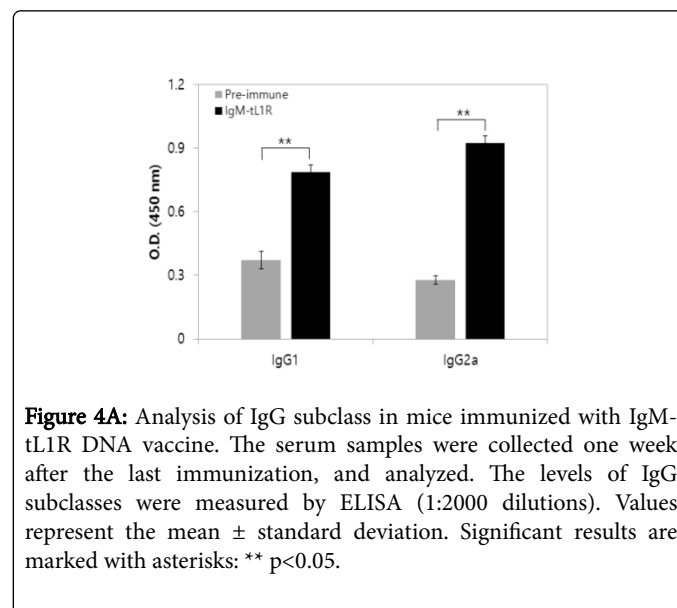

Figure 4A: Analysis of IgG subclass in mice immunized with IgM-tL1R DNA vaccine. The serum samples were collected one week after the last immunization, and analyzed. The levels of IgG subclasses were measured by ELISA (1:2000 dilutions). Values represent the mean ± standard deviation. Significant results are marked with asterisks: ** $p < 0.05$.

Discussion and Conclusion

The threats of biological warfare and bioterrorism may require the vaccination of large populations against smallpox. Although the present licensed smallpox vaccine induces a robust immune response, it has severe adverse reactions. In addition, a considerable amount of time is required to produce sufficient quantities of the vaccine. Therefore, new safer immunization strategies are needed to prepare for emergency smallpox vaccination. Given these considerations, we developed and tested plasmid-based vaccine candidate for smallpox.

L1 is a transmembrane protein of 250 residues that has an N-terminal ectodomain (1-185) region and a C-terminal hydrophobic region (186-204), which is embedded in IMV viral membranes. Full-length L1 has been included as an essential component of multicomponent vaccines that provided protection against lethal vaccinia virus challenge. Recently it has been suggested that protein- or

DNA-based vaccines using a truncated form of L1, consisting of the expressed ectodomain, can induce a potent neutralizing antibody response [28,29]. However, only partial protection against disease was conferred by this variant. In this report, we present evidence that truncated and modified L1R is sufficient to not only produce high levels of neutralizing antibodies but also to protect mice from lethal intranasal challenge with virulent vaccinia virus.
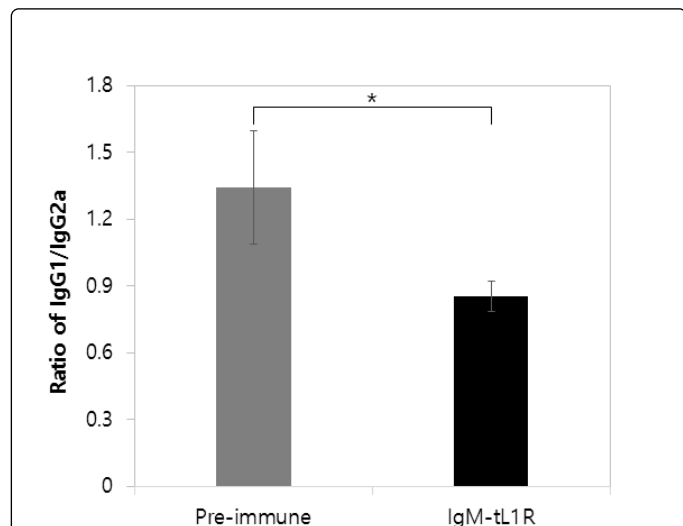

Figure 4B: IgG1 to IgG2a antibody ratios are shown in immunized and pre-immunized group. Significant results are marked with asterisks: * p<0.5.

Since previous DNA vaccine studies in our laboratory indicated that strategies, including codon optimization, addition of a signal sequence, and inclusion of transcriptional enhancer, were able to induce a strong protective immune response against anthrax spore challenge, in this study, similar approaches were performed to develop smallpox DNA vaccine. Codon optimization is beneficial to DNA vaccine development, probably by sufficient antigen expression in the host cells. Barrett et al. suggested that poxvirus genomes that are high-AT content in the 3rd position of the codon, including orthopoxviruses, would be predicted to be an inefficient expression in mammalian cells, and this problem could be overcome by codon optimization [30]. Furthermore, a codon-optimized vaccinia virus A27L gene was recently reported to elicit an enhanced immune response [31]. In our experiments, the original L1R and codon-optimized tL1R were 50% different in terms of codon usage (data not shown). Previous poxvirus DNA studies demonstrated that signal sequence was able to promote the secretion of antigens and their immune responses [32,33]. These studies indicated that a tissue plasminogen activator signal peptide fused to L1R induced a higher amount of neutralizing antibodies and provided better immunogenicity than unmodified L1R. Our results showed that tL1 protein, which contains IgM signal peptide sequence with lacking transmembrane domain, was secreted into the extracellular space. Besides antigen optimization, we also employed an optimized DNA vaccine vector carrying a transcriptional enhancer that was able to strengthen the antigen production and therefore immune response of DNA vaccine. Recently our studies revealed that inclusion of the tandem repeat sequence of SV 40 enhancer upstream of a promoter in the plasmid vector enhanced the antibody production as much as 10-fold [19].
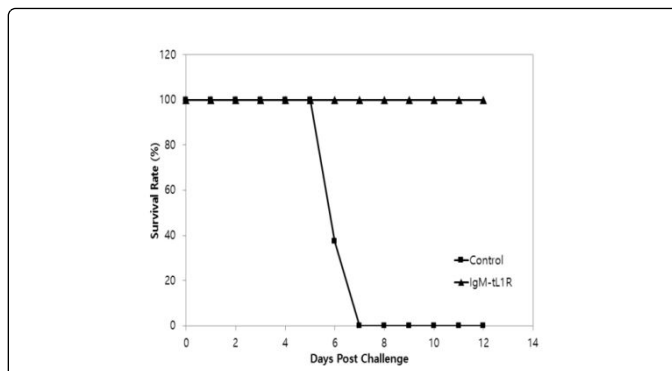

Figure 5A: Survival of BALB/c mice immunized with IgM-tL1R DNA vaccine after vaccinia virus challenge. Mice (n=5/group) were immunized three times with 50 µg of IgM-tL1R DNA vaccine at 2-week intervals. Vaccinated mice were challenged intranasally with vaccinia virus (1×10^6 PFU) two weeks after the final immunization. Control group mice were infected but were treated with vector only. Survival curves show the percentage of mice alive after challenge.
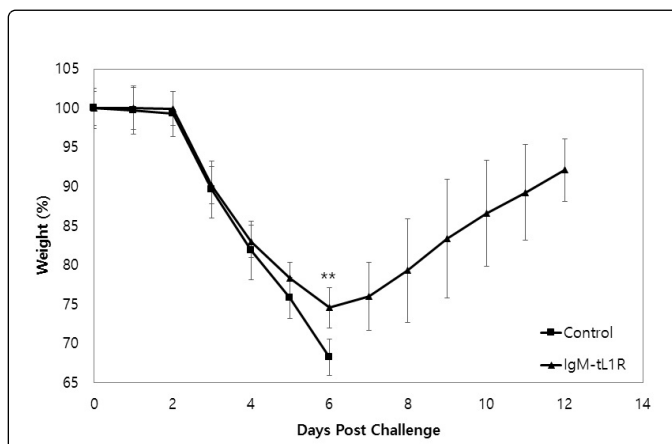

Figure 5B: Body weight loss as the percentage of pre-challenged weight was plotted. Values represent the mean ± standard deviation. Significant results are marked with asterisks: ** p<0.05.

Following immunization with IgM-tL1R through electroporation, we observed the induction of a prominent antibody response. At five weeks post-immunization, the antibody titer was approximately 3.0 log titers which were similar to other studies using a modified L1R construct [28,33]. The capacity tL1R-specific antibodies to neutralize vaccinia virus was also confirmed by plaque assay. A strong neutralizing immune response was elicited, based on serum samples from the group immunized with IgM-tL1R. Although the pre-immune group produced some level of neutralizing activity in our experiments, it seemed that complement protein might have contributed to this phenomenon [34]. It is generally thought that intrinsic, nonspecific neutralization present in mice sera. Jayasekera et al. have shown that nonimmune serum neutralized influenza virus, suggesting that coating of virions with complement proteins can neutralize virus by nonspecifically blocking virus receptors [35]. One of the advantages of a DNA vaccine is its ability to induce strong cell-mediated immune

responses. Both IgG1 and IgG2 were detected after immunization indicating that both Th1 and Th2 immune systems were stimulated. However, the resulting IgG1/IgG2a ratio was <1.0, suggesting that the Th1 response was more prominent, which indicates that IgM-tL1R elicited a cell-mediated immune response. Indeed, induction of both Th1- and Th2- mediated immune response by IgM-tL1R will offer advantages for protection against orthopoxvirus infection.

In this report, we showed that a high level of protection against vaccinia virus was provided by vaccination with IgM-tL1R. Protection was observed at 50 LD_{50}. However, despite this high level of protection, transient weight loss after the challenge was observed in mice immunized with IgM-tL1R. These results indicate that although our modification of previous L1R DNA vaccine with the addition of enhancer sequences and electroporation delivery provides the protection from mortality, further optimization is necessary to reduce morbidity.

Our study is comparable to previous work [28]. Shinoda et al. showed that immunization with codon optimized DNA vaccine that express L1R containing Ig κ-chain signal peptide sequence elicited antibodies that were able to provide partial protection of mice from 10^4 PFU challenge with vaccinia virus. However, it is not possible to directly compare the studies, since we used electroporation, whereas before other group used gene gun for immunization. Previously, it was suggested that different application methods of DNA vaccines have profound effects on immunogenicity [22].

Taken together, our results may contribute to the development safe and efficacious smallpox DNA vaccine to protect against potential biological warfare and bioterrorism attack using smallpox.

Acknowledgments

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